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SHB1 plays dual roles in photoperiodic and autonomous flowering

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ABSTRACT

Flowering was initiated by the integration of environmental signals such as day-length with the internal development status in *Arabidopsis*, a facultative long-day plant. The photoperiodic flowering involves two key components, CONSTANS and FT, whereas the autonomous flowering is operated through a central quantitative floral repressor, FLC, and several other genes that act upstream of FLC. SOC1 acts downstream to integrate the flowering signals from the two pathways. Here, we report that SHB1 plays dual roles in both photoperiodic and autonomous flowering. *shb1-D*, a gain-of-function mutant, flowered early and *shb1*, a loss-of-function allele, flowered late under both long days and short days. The *shb1-D* mutation activated the expression of *CO*, *FT*, and *SOC1* under both long and short days, and however, the *co-2* mutation attenuated the *shb1-D* activated expression of *FT* and *SOC1* only under long days but not short days. The *shb1-D* or *shb1* mutations also reduced and increased, respectively, the expression of *FLC* under both long and short days. Transgenic remedy of FLC to wide-type level in *shb1-D* background also reverted *shb1-D* flowering and *FT* or *SOC1* expression to wild type mostly under short days. Furthermore, the *shb1-D* suppression on *FLC* expression is likely operated through LD as *ld-3* blocked this suppression and SHB1 appears to act upstream of LD. In summary, SHB1 represents signaling steps that regulate *CO* expression in leaves and *LD* or *FLC* expression in either leaves or shoot apical meristem, contributing to a threshold expression of *SOC1* in shoot apical meristem for floral initiation.

Introduction

Plant flowering is controlled by the integration of environmental signals with the developmental status of a plant (Searle and Coupland, 2004). The exogenous regulations include day-length sensing, light quality perception, and temperature response or vernalization. The internal signaling cascades in response to certain developmental stages or leaf numbers are defined as autonomous pathway. In addition, gibberellic acid (GA) pathway seems to act independently of either exogenous or autonomous regulation (Mouradov et al., 2002; Boss et al., 2004). *Arabidopsis* is a facultative long-day plant, and its flower initiation is accelerated under long days but delayed under short days (Searle and Coupland, 2004).

CONSTANS (*CO*) encodes a zinc finger protein and CO plays a key role in the photoperiodic flowering pathway (Putterill et al., 1995). *CO* expression is under the control of the circadian clock and is also regulated by different light wavelengths (Imaizumi et al., 2003, 2005). The abundance of *CO* mRNA was reduced in *phyA* but was slightly increased in *phyB* under long days (Cerdán and Chory, 2003; Putterill et al., 1995; Tepperman et al., 2001). The expression of *FT* was also reduced in the *phyA* and *cry2* mutants under long days (Yanovsky and Kay, 2002). In addition, CO protein is subject to posttranscriptional regulation by light signals of various wavelengths (Valverde et al., 2004). crys and phyA stabilize CO protein under blue or far-red light, whereas phyB promotes the degradation of CO under red light, generating a daily rhythm in the abundance of CO. CO directly controls the expression of *FT* (*FLOWERING LOCUS T*) or *SOC1* (*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*) (Cerdán and Chory, 2003; Samach et al., 2000; Suárez-López et al., 2001; Yanovsky and Kay, 2002). SOC1 acts further downstream and integrates signals from several flowering pathways.

The autonomous pathway mutants display photoperiod-independent late flowering and strong acceleration of flowering in response to prolonged exposure to cold (Koornneef et al., 1991). The central quantitative floral repressor FLC (FLOWERING LOCUS C), a MADS-domain transcription factor, integrates vernalization response and autonomous regulation in *Arabidopsis* (Michaels and Amasino, 1999; Sheldon et al., 1999; Michaels and Amasino, 2001). FLC represses flowering, at least partly, by directly binding to specific regulatory elements in the *FT* and *SOC1* loci (Hepworth et al., 2002; Helliwell et al., 2006; Searle et al., 2006). Several genes upstream of *FLC* in this pathway include *FVE*, *FLD*, *LD* (*LUMINIDEPENDENS*), *FLK*, *FY*, *FCA* and *FPA* (Koornneef et al., 1991; Lee et al., 1994; Chou and Yang, 1998; Schomburg et al., 2001; Lim et al., 2004; Mockler et al., 2004). *ld-3* mutant is late flowering in both long and short days but the delay of flowering is more extreme in short days (Lee et al., 1994). The flowering of the *ld* mutants can be accelerated by vernalization in both

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long days and short days. LD is a nuclear protein and negatively regulates the expression of *FLC* along with other autonomous pathway components (Michaels and Amasino, 1999). The expression of *FLC* was increased in *ld* mutant, and this expression change was correlated with a strong increase in trimethylation and Histone acetylation of the *FLC* locus (He and Amasino, 2005; Domagalska et al., 2007).

SHB1 was initially isolated for its role in light-mediated hypocotyl elongation response (Kang and Ni, 2006). *SHB1* encodes a yeast SYG1-like protein with a conserved N-terminal SPX motif and a C-terminal EXS motif (Kang and Ni, 2006). Different from other members in the SYG1 family (Spain et al., 1995), SHB1 is localized into the nucleus. SHB1 is also involved in the regulation of seed development (Zhou et al., 2009). In this study, we report the function of SHB1 in *Arabidopsis* flowering. SHB1 positively regulates the expression of *CONSTANS* and the signal is further propagated to increase the expression of *FT* and *SOC1* particularly under long days**.** Under short days, SHB1 activates the expression of *LD* to suppress the expression of *FLC* but to allow the activation of *SOC1* in shoot apical meristem. Therefore, SHB1 plays dual roles in both the day-length pathway and the autonomous pathway and may define a signal step that the two flowering pathways interact.

Materials and methods

Plant materials and flowering experiments

shb1 (SALK_128406) in Columbia (Col) background and *ld-3* in Wassilewskija (Ws) background were obtained from the *Arabidopsis* Biological Resources Stock Center (Ohio State University, Columbus). *shb1-D* was isolated in Ws background and the *SHB1* overexpression lines in Ws and Col background were generated as described previously (Kang and Ni, 2006). *flc-3* is in the Columbia background as described previously (Michaels and Amasino, 1999). *phyB-9*, *phyA-211*, *cry1-304*, *cry2-1*, and *co-2* were described previously (Kang et al., 2007). *shb1-D* and *shb1* mutants were backcrossed twice to wild type before phenotypic analysis.

Flowering experiments were conducted under either long days (16 h light/8 h dark) or short days (8 h light/16 h dark) with fluorescent cool white light at 22 °C. Flowering time was determined as rosette leaf numbers after bolting 1 cm. For analysis of *CO*, *FT*, *SOC1*, *FLC* and *LD* expression, Ws, *shb1-D*, Col, and *shb1* seedlings were grown under long days or short days for 12 days and sampled at Zeitgeber times 12 and 16 (LD) or 8 and 12 (SD) when *CO* and *FT* have the highest levels of expression.

Gene expression and real-time RT-PCR analysis

For qRT-PCR analysis, total RNAs were isolated by using the SV total RNA isolation kit (Promega). SuperScript™ III reverse transcriptase (Invitrogen) was used to synthesize the first-strand cDNA with oligo dT primer (or random hexamers from Thermo Scientific, UK for the analysis of *FLC* expression in *FLC* transgenic plants that carry endogenous *FLC* and *FLC* transgene) and 1 μg of total RNA at 50 °C for 1 h. Quantitative PCR was then performed with Platinum SYBR Green qRT-PCR kit (Invitrogen) on Applied Biosystems 7500 Real-Time PCR machine. The thermal cycling program was 50 °C for 10 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min and a one-cycle dissociation stage at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The primer pairs used in qRT-PCR were *FT*, 5′-GAGACCCTCTTATAGTAAGCA-3′ and 5′-CTTCCTCCGCAGCCACTCA-3′; *SOC1*, 5′-AATATGCAAGATACCATAGATCGT-3′ and 5′-TTCTTGAAGAACAAGGTAACCCAA-3′; *CO*, 5′-ACGCCATCAGCGAGTTCC-3′ and 5′-AAATGTATGCGTTATGGTTAATGG-3′; *FLC*, 5′-AGTAGCCGACAAGTCACCTT-3′ and 5′-GAGAGTCACCGGAAGATT-3′; *LD*, 5′-AACAGCAACAATATATGCAAC-3′ and 5′-ATATCCTGGATTGCTACTCAT-3′; *UBQ10*, 5′-AGGTACAGCGAGAGAAAGTAGCA-3′ and 5′-TAGGCATAGCGGCGAGGCGT-3′. Data were calculated from three biological samples and each biological sample was examined in triplicate.

For tissue-specific expression analysis, different tissue types were harvested from plants and frozen immediately in liquid nitrogen, including 5-day-old light-grown seedlings, inflorescence meristem and shoot apex, closed floral buds and flowers of different stages, siliques at 1 to 8 days after pollination, rosette and cauline leaves of sizes 5 to 12 mm, and internodes from 4-week-old plants. Roots were harvested from seedlings at 12 days after germination. Primer pairs used for *SHB1* expression were 5′-CAGGTTCAAGCACTGAGGAGT-3′ and 5′-TGCTTCCTCGGTTTAGAGTA-3′.

Transgenic expression of FLC in shb1-D

FLC cDNA was PCR-amplified and subcloned into the Xho I and BamH I sites of pEZT-NL binary vector. This construct was transformed into *shb1-D* through floral dip method by using *Agrobacterium* strain AGl1. More than 50 F1 transgenic plants were screened and homozygous lines were identified from F2 population based on their Basta resistance. Total RNAs from *shb1-D* transgenic plants and *shb1-D* were isolated and the levels of *FLC* transcript were examined through RT-PCR analysis.

Double mutant analysis

To control the difference in flowering responses between different ecotypes, the entire F2 population was scored for flowering times under long days and short days and each individual was PCR-genotyped. Mean values plus or minus standard errors for each genotype from the entire F2 population were calculated. To generate *shb1-D*/*co-2* double mutants, *shb1-D* (Ws) was crossed to *co-2* (L*er*). Gene specific primer pairs for *shb1-D* genotyping were 5′-GAAGATACGGGTTTTGCAT-3′ and 5′-GGGAAGCTTGGATGTCTTGAA-3′. The T-DNA specific primer for *shb1-D* genotyping was 5′-CATTTTATAATAACGCTGCGGACATCTAC-3′. The *co-2* locus contains a point mutation and was genotyped by using two pairs of length polymorphic markers closely linked to the *co-2* locus (Kang et al., 2007). To analyze the expression of *FT* and *SOC1* in the double mutants, F3 seedlings derived from several homozygous double mutants, single mutants or wild types, all in mixed background, were grown under either long days or short days for 12 days, and were sampled at Zeitgeber time 16 (LD) or 12 (SD).

shb1/*flc-3* was generated by crossing *shb1* (Col) to *flc-3* (Col) and *flc-3* mutation was genotyped according to the procedure as previously reported (Lee et al., 2000; Moon et al., 2005). *shb1*/*phyB-9* was generated by crossing *shb1* (Col) to *phyB-9* (Col). The *shb1* locus was PCR-genotyped and gene specific primer pairs for *shb1* genotyping were 5′-TAAGCAGCACGAGCTCAAAT-3′ and 5′-TGCTTCCTCGGTTTAGAGTA-3′. The T-DNA specific primer for *shb1* genotyping was 5′-GGAACCACCATCAAACAGGAT-3′. The F2 populations for *shb1-D*/*phyB-9*, *shb1-D*/*phyA-211*, *shb1-D*/*cry2-1*, and *shb1*/*cry2-1* double mutants were generated previously (Kang and Ni, 2006). The *phyB-9* or *phyA-211* mutation was back-genotyped in the F3 generation for its dramatic hypocotyl phenotype under red or far-red light. *cry2-1* contains large deletions and homozygous *cry2* mutation was genotyped by using the following pair of primers: 5′-GGTTTAGAAGAGACCTAAGGAT-3′ and 5′-CCAGATTCTTCCCTTCTGAT-3′.

Chromatin immunoprecipitation or ChIP analysis

ChIP analysis was performed as previously described (Zhou et al., 2009). Seedlings of 11-day-old (about 2 g) were used for preparation of each biological replicate. Quantitative PCR was performed as described under the section of real-time PCR analysis. The primer pairs used in real-time PCR experiments were: *CO-1*, 5′-TCGAGTGTCAGAGCCATCAC-3′ and 5′-TTCATGTCACTTTTCCGATAT-3′; *CO-2*, 5′-

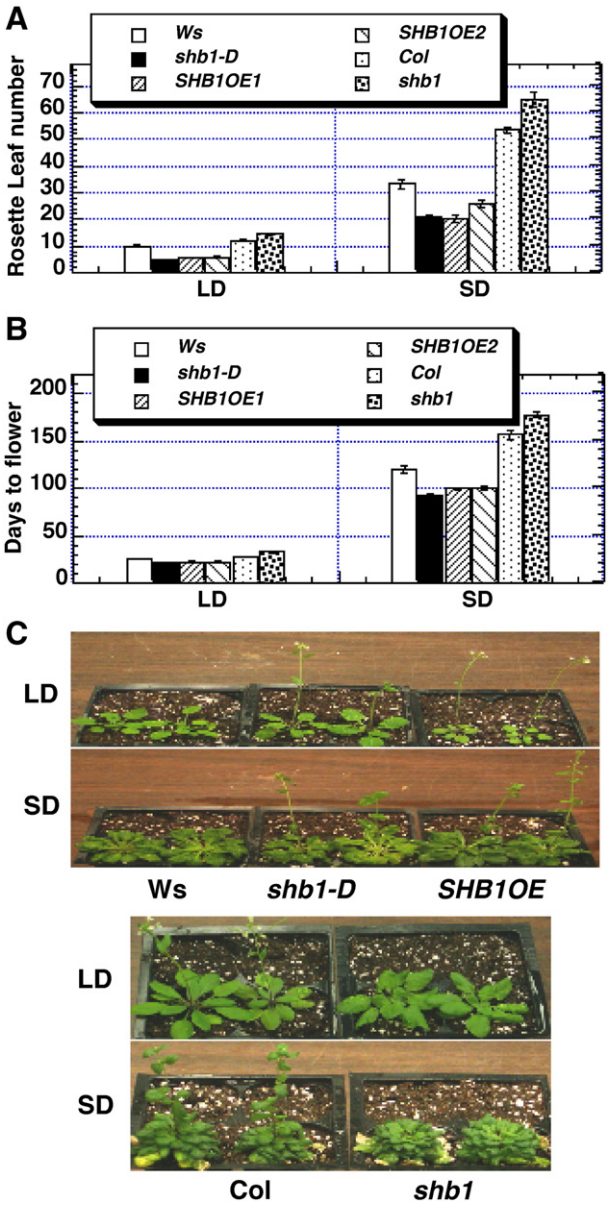

Fig. 1. *shb1* mutations affect flowering. (A) Rosette leaf numbers of Ws, *shb1-D*, and the transgenic plants that overexpress full-length SHB1 (*SHB1 OE*), Col, and *shb1* under LD (16 h light/8 h dark) or SD (8 h light/16 h dark). (B) Days to flowering of Ws, *shb1-D*, *SHB1 OE*, Col, and *shb1* under LD or SD. Means plus or minus the standard errors were calculated from at least 25 plants. (C) Ws, *shb1-D*, and *SHB1 OE* at 30 days after germination under LD or at 100 days after germination under SD (upper), and Col and *shb1* at 35 days after germination under LD or at 140 days after germination under SD (lower).

TATGGTCCCTCGACTCTTATT-3′ and 5′-GCCTTCGGATAACTGTTACGA-3′; *LD-L1*, 5′-TATTATCACCCAAATCAAAC-3′ and 5′-TTCGTCTTTCTAGGTTTTTA-3′; *LD-L2*, 5′-TGATACCTCGAGATCTTAAA-3′ and 5′-ATAGGTCTCGTCTCTCTTTC-3′; *UBQ10*, 5′-TCCAGGACAAGGAGGTATTCCTCCG-3′ and 5′-CCACCAAAGTTTTACATGAAACGAA-3′.

Results

shb1 mutations affect flowering under both long days and short days

To explore if SHB1 also acts in other phases of *Arabidopsis* development, we examined the flowering phenotypes of *shb1-D* and *shb1*. Compared to Ws wild type, *shb1-D* and *SHB1* overexpression transgenic plants flowered early under both long days and short days and these plants produced fewer rosette leaves compared to wild type at bolting (Fig. 1A). By contrast, *shb1*, a loss-of-function allele, flowered late compared to Col wild type in both long days and short days (Fig. 1A).

shb1 mutations affect the expression of two key photoperiodic flowering genes

We next examined the expression of two key photoperiodic flowering pathway genes, *CO* and *FT*, by real-time RT-PCR analysis (Fig. 2). The seedlings were sampled at Zeitgeber time (ZT) 12 and 16 in long days and 8 and 12 in short days when *CO* and *FT* were expressed at their peaks (Searle et al., 2006; Wigge et al., 2005). At ZT 16 under long days, the expression of *CO* level was increased 2-fold in *shb1-D* and repressed 2-fold in *shb1* compared to wild type (Fig. 2A). The expression of *FT*, a gene immediately downstream of CO, at ZT 16 h under long days showed a similar scale of changes as that of *CO* in *shb1-D* and *shb1* mutants compared to wild type (Fig. 2B). We also examined the expression of *SOC1*, a read-out gene further downstream of both photoperiodic and autonomous flowering pathways. At ZT 12 h and 16 h under long days, the expression of *SOC1* was enhanced 2.5-fold in *shb1-D* and repressed 2-fold in *shb1* compared to wild type (Fig. 2C).

At ZT 12 h under short days, the *shb1* mutations also caused a 2-fold increase or decrease in *CO* expression (Fig. 2A). In contrast, at ZT 8 under short days, the expression of *FT* was enhanced up to 6

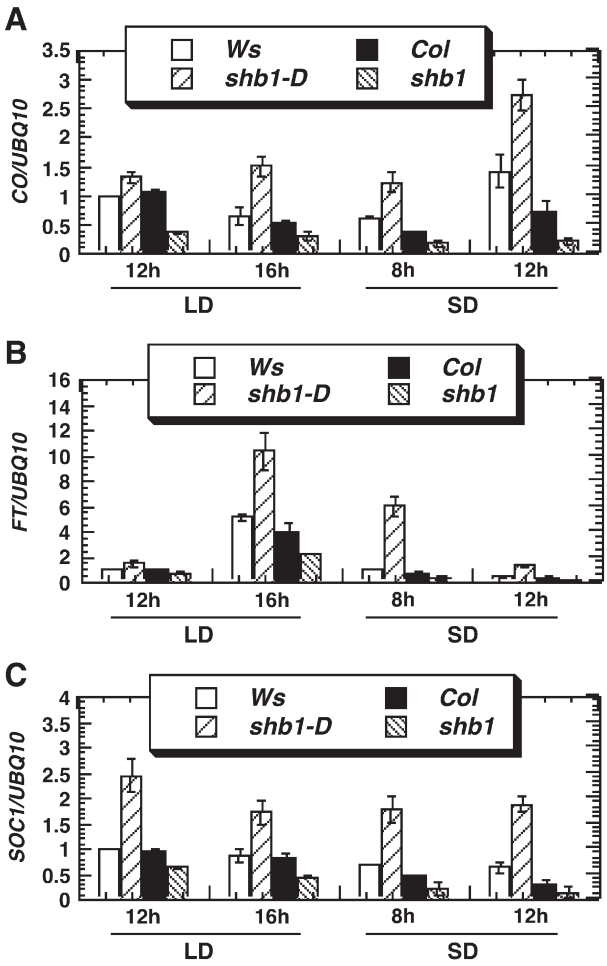

Fig. 2. *shb1* mutations affect the expressions of *CO* (A), *FT* (B), and *SOC1* (C) under long days and short days as analyzed through real-time RT-PCR. The expression of each gene is normalized to that of Ws at ZT 12 h under long days except for the expression of *FT* in short days, which is normalized to that of Ws at ZT 8 h under short days. Data are presented as means plus or minus the standard errors from three biological samples and each biological sample was examined in triplicate.

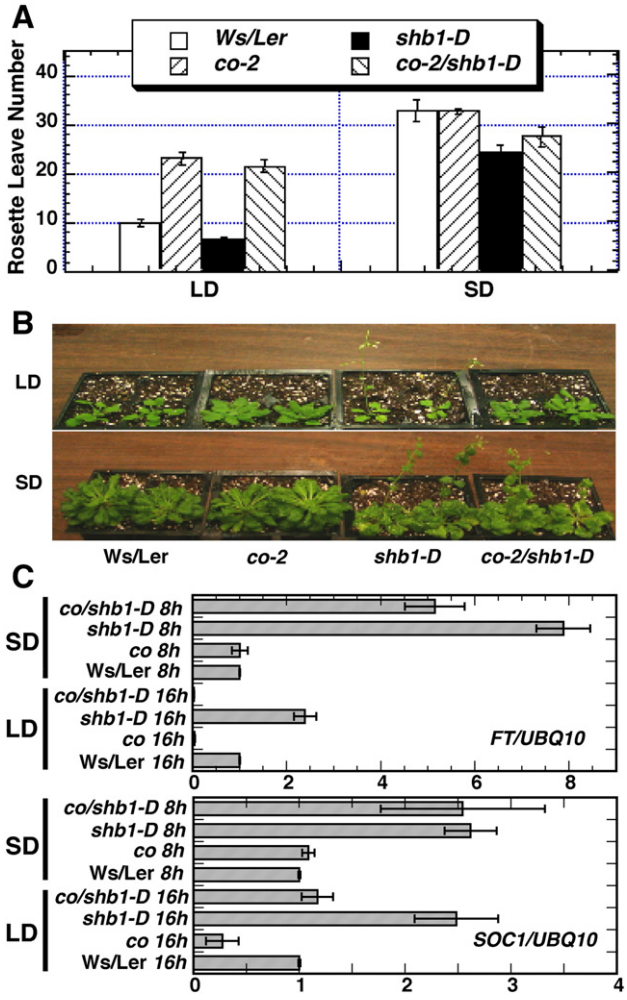

Fig. 3. *co-2* is epistatic to *shb1-D* under long days. (A) Rosette leaf number of wild type (Ws/Ler), *co-2* (Ws/Ler), *shb1-D* (Ws/Ler), and *shb1-D*/*co-2* (Ws/Ler) when bolting 1 cm. At least six independent lines from each genotype in a mixed Ws/Ler background were used in the calculation. Data are presented as means plus or minus the standard errors. (B) Ws/Ler, *co-2* (Ws/Ler), *shb1-D* (Ws/Ler), and *shb1-D*/*co-2* (Ws/Ler) at 30 days after germination under LD (upper) or at 90 days after germination under SD (lower). (C) Expression of *FT* (upper) and *SOC1* (lower) in Wild type (Ws/Ler), *co-2* (Ws/Ler), *shb1-D* (Ws/Ler), and *shb1-D*/*co-2* (Ws/Ler) under LD and SD. Data are presented as means plus or minus the standard errors from three biological samples and each biological sample was examined in triplicate.

times in *shb1-D* and repressed more than 3 times in *shb1* (Fig. 2B). Such changes may not be as significant since the level of *FT* transcript is not very abundant under short days. The expression of *SOC1* was enhanced 2.5-fold by *shb1-D* and repressed 2-fold by *shb1* at ZT 8 and 12 under short days (Fig. 2C). As previously reported, the level of CO protein is only easily detectable in the nuclear protein extracts of 35S:: CO plants at its accumulation peak ZT12 under dark/light cycle, and CO protein is most abundant under blue light (Valverde et al., 2004). We barely detected CO protein in total protein extracts prepared from wild type and *shb1* mutants under dark/light cycle. We then prepared total protein extracts from plants that were grown under long days for 10 days, transferred to blue light at ZT 0 in the morning, and sampled at ZT 12. This experimental condition allowed us to detect CO protein but not quantitatively, and the levels of CO protein were not significantly altered in Ws and *shb1-D*, suggesting that SHB1 is not involved in blue light-mediated CO protein stability (data not shown). Considering the fact that we only detected a 2-fold or more change of CO transcript in *shb1* mutants compared to wild type through qRT-PCR analysis under dark/white light cycle, the current technique and conditions to follow CO protein exclude us to detect a parallel change in CO protein level.

shb1-D enhances flowering through CO and FT in long days

To test if the change in the expression of *SOC1*, a more reliable downstream read-out of the flowering response, is due to altered expression of *CO* and *FT* by *shb1-D* under long days, we constructed double mutant of *shb1-D* (Ws) with *co-2* (*Ler*). We examined the flowering response and the expression of *FT* and *SOC1* in Ws, *shb1-D*, *Ler*, *co-2*, Ws/*Ler*, and *shb1-D*/*co-2* (Fig. 3). We selected *shb1-D* since *shb1-D* has a stronger flowering phenotype than that of *shb1* and the flowering phenotype of *shb1-D* is opposite to that of *co-2*. To control the difference in flowering responses between crosses of different ecotypes, we determined the flowering responses of various genotypes in a segregating F2 population. *shb1-D* flowers early under both long days and short days, whereas *co-2* flowers late under long days but not under short days (Figs. 1, 3). The *shb1-D*/*co-2* double mutant flowered as late as single *co-2* mutant under long days, but flowered as single *shb1-D* mutant under short days (Figs. 3A, B).

Since the flowering response of *shb1-D* under long days is mainly operated through the CO signaling branch, *co-2* mutation may strongly block the activated expression of *FT* or *SOC1* by the *shb1-D* mutation. At ZT 16 in long days, the activation of *FT* expression by the *shb1-D* mutation was largely impaired by the *co-2* mutation, however, at ZT 8 in short days, the activation of *FT* expression in *shb1-D* was only partially affected by the *co-2* mutation (Fig. 3C). At ZT 16 in long days, the activation of *SOC1* by *shb1-D* mutation was also significantly suppressed by the *co-2* mutation (Fig. 3C). However, at ZT 8 in short days, the activated expression of *SOC1* by the *shb1-D* mutation was barely affected by the *co-2* mutation (Fig. 3C). Therefore, the function of SHB1 in floral initiation and the activation of *FT* or *SOC1* expression requires a functional CO in long days but is barely dependent on the function of CO in short days.

SHB1 acts in autonomous pathways and negatively regulates FLC expression

As the activated expression of *SOC1* in *shb1-D* was not significantly affected by the *co-2* mutation under short days, the flowering response of *shb1-D* may involve other components in vernalization and autonomous pathway such as FLC, a major floral repressor in *Arabidopsis*. We thus examined the expression of *FLC* in *shb1* mutants by qRT-PCR (Fig. 4A). The expression of *FLC* was suppressed by 2-fold in *shb1-D* and increased 2-fold in *shb1* under both long days and short days. The expression of *FLC* is tightly regulated and this quantitative nature makes it a very sensitive indicator of the flowering response. For example, overexpression of *FLC* strongly suppressed the expression of both *FT* and *SOC1* and floral initiation (Hepworth et al., 2002; Michaels et al., 2005).

If the FLC signaling branch plays a minor role for the early flowering of *shb1-D* under long days, the expression of *FLC* at or slightly above the wild type level may not affect the activation of *SOC1* expression by the *shb1-D* mutation. In contrast, CO may play a major role in long days but not short days, and the expression of *FT* and *SOC1* may be mainly operated through the FLC signaling branch in short days. Therefore, the expression of *FLC* at or slightly above the wild type level in the *shb1-D* background could largely block the early flowering response of *shb1-D* as well as the activation of *FT* or *SOC1* expression under short days. We generated several transgenic lines that overexpress *FLC* in the *shb1-D* mutant background, and two lines had a comparable level of *FLC* transcript to that of the wild type (Fig. 4B). A third line had a higher level of *FLC* transcripts than that of wild type (Fig. 4B).

We then examined the flowering responses of these transgenic lines under both long days and short days (Fig. 4C). The two transgenic lines with *FLC* expressed at the wild type level in the *shb1-D* background showed a *shb1-D*-like early flowering phenotype under long days, but a wild type-like flowering phenotype under short days

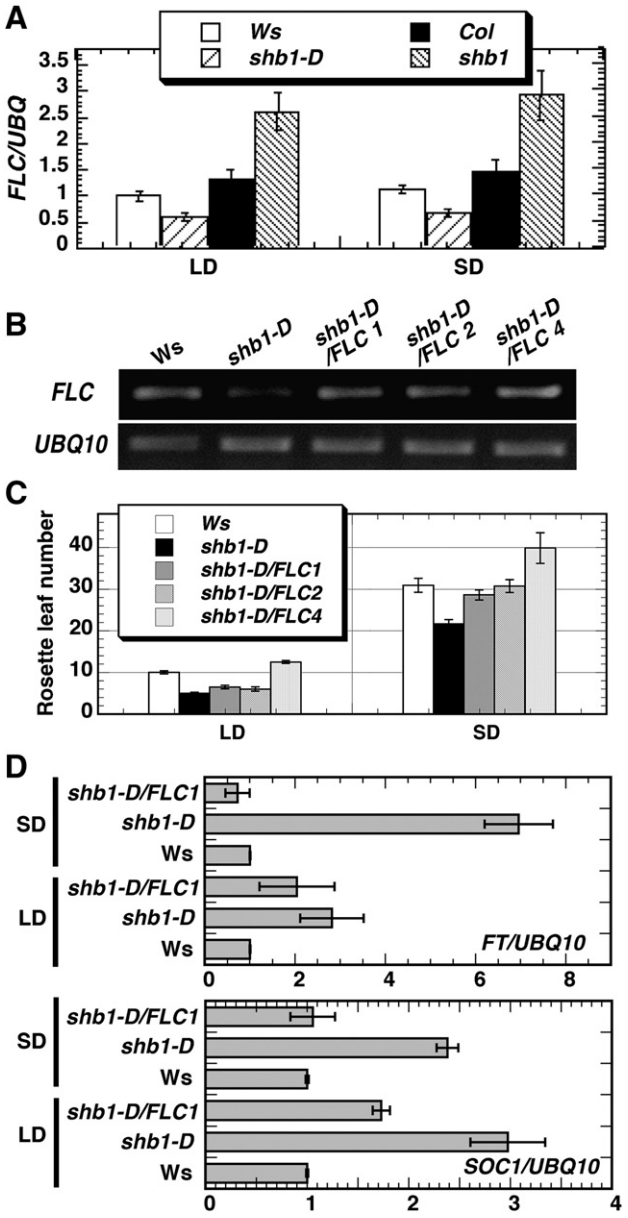

Fig. 4. *shb1* mutations affect the expression of *FLC*. (A) Real-time RT-PCR analysis of *FLC* expression in Ws, *shb1-D*, Col, and *shb1* under LD and SD. (B) Expression of *FLC* in Ws, *shb1-D*, and several transgenic lines that carry a *FLC* transgene in *shb1-D* background, including the endogenous *FLC* transcripts and the transcripts of the *FLC* transgene. (C) Rosette leaf number of Ws, *shb1-D*, and several transgenic lines that carry a *FLC* transgene in *shb1-D* background under LD and SD. Means plus or minus the standard errors were calculated from at least 15 plants. (D) Expression of *FT* and *SOC1* under LD and SD in Ws, *shb1-D*, and a transgenic line that carries a *FLC* transgene in *shb1-D* background. The expression of each gene is normalized to that of Ws wild type under LD. Expression data are presented as means plus or minus the standard errors from three biological samples and each biological sample was examined in triplicate.

(Fig. 4C). The third line that overexpresses *FLC* above the wild type level in the *shb1-D* background showed a late flowering phenotype under long days, but a much late flowering phenotype under short days (Fig. 4C). The activation of *FT* expression by the *shb1-D* mutation was barely affected in the two transgenic plants that express *FLC* at the wild type level at ZT 16 in long days, but was significantly reduced compared to *shb1-D* at ZT 12 in short days (Fig. 4D). Furthermore, the activation of *SOC1* expression by the *shb1-D* mutation in the two transgenic plants that express *FLC* at the wild type level was partially suppressed under long days, but suppressed to the wild type level under short days (Fig. 4D). FLC apparently plays a more prominent role to mediate *shb1-D* activation under short days, whereas CO may play a dominant role downstream of SHB1 under long days.

We also constructed *shb1*/*flc-3* double mutant and examined their flowering responses under long days and short days. Under long days, *shb1* flowered late and *flc-3* flowered slightly earlier than Col as reported previously by others (Fig. 5A, Michaels and Amasino, 1999). The *flc-3* mutation only partially suppressed the late flowering phenotype of *shb1* under long days, but was completely epistatic to *shb1* under short days and *shb1*/*flc-3* showed a *flc-3* like early flowering phenotype (Fig. 5A). The *flc-3* mutation also significantly altered the effects of the *shb1* mutation on the expression of either *FT* or *SOC1* under short days, but only partially altered the effects of the *shb1* mutation on the expression of *FT* and *SOC1* under long days (Figs. 5B, C).

SHB1 acts upstream of LD

We also examined the expression of all known genes in autonomous flowering pathway upstream of *FLC* in either *shb1-D* or *shb1* compared to wild type through RT-PCR analysis. SHB1 regulated the expression of *LD* positively but not any other genes characterized so far in the pathway under long days and short days (Fig. 6A; data not shown). The expression of *LD* was increased 2-fold by the *shb1-D* mutation compared to wild type and was reduced by half in *shb1* compared to wild type (Fig. 6A). LD has been shown as a positive regulator upstream of FLC in the autonomous flowering pathway, and *ld-3* mutant flowered late and the expression of *FLC* is up-regulated in

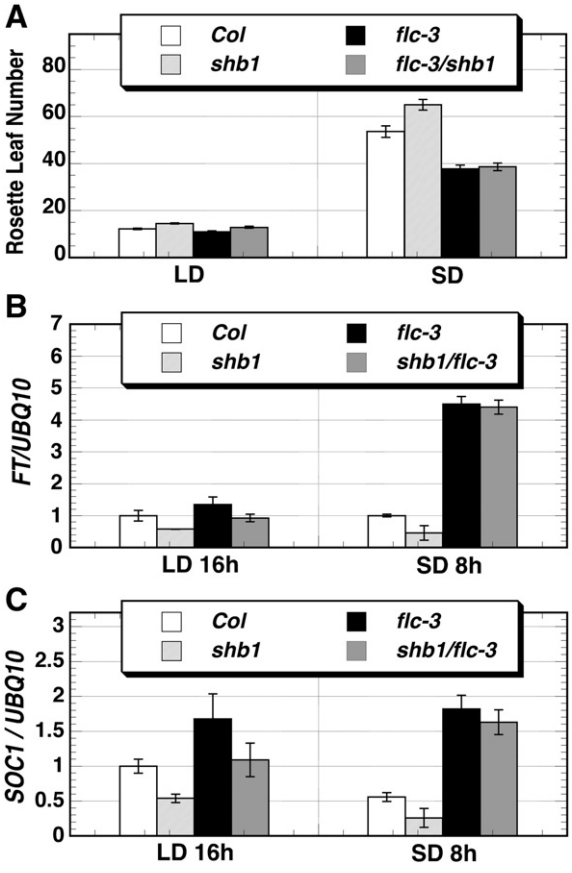

Fig. 5. SHB1 acts upstream FLC. (A) Rosette leaf number of Col, *shb1*, *flc-3*, and *shb1*/*flc-3* under LD and SD. Means plus or minus the standard errors were calculated from at least 15 plants. (B) Expression of *FT* and *SOC1* (C) in Col, *shb1*, *flc-3*, and *shb1*/*flc-3* under LD and SD as analyzed through real-time RT-PCR. The expression of each gene is normalized to that of Ws wild type at ZT 12 h under LD except for the expression of *FT* under SD, which is normalized to that of Ws wild type at ZT 8 h under SD. Expression data are presented as means plus or minus the standard errors from three biological samples and each biological sample was examined in triplicate.

ld-3 (Koornneef et al., 1991; Michaels and Amasino, 1999). Interestingly, the tissue-specific expression pattern of *SHB1* overlaps with that of *LD*. *LD* transcript and protein are very abundant in shoot apices, floral bus, stems, and roots, and less abundant in leaves (Aukerman et al., 1999). The expression of *SHB1* was observed strong in flower buds (including floral organs, pollen grains, and pre-pollinated ovules), intermediate in seedlings, shoot apex, and developing cliques, and weak in cauline leaves (Fig. 6B).

We further conducted double mutant analysis and found that *ld* is epistatic to *shb1-D* and *shb1-D*/*ld-3* double mutant flowered as *ld-3* did under both long days and short days (Fig. 6C). The expression of *FLC* in the *shb1-D*/*ld-3* double mutant was similar to that of the *ld-3* single mutants under either long days or short days (Fig. 6D).

SHB1 function requires photoreceptors

To learn the function of SHB1 in photoperiodic flowering with respect to the photoreceptors, we studied the genetic interaction of

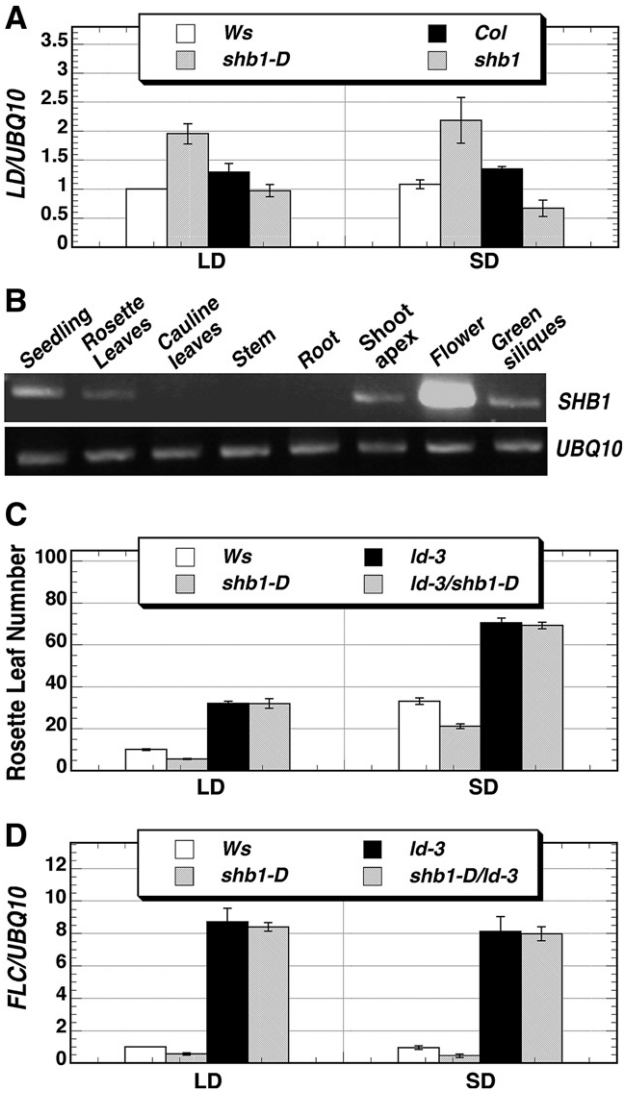

Fig. 6. SHB1 acts upstream of LD. (A) Real-time RT-PCR analysis of *LD* expression in Ws, *shb1-D*, Col, and *shb1* under LD and SD. (B) Expression of *SHB1* in various tissue or organ types as analyzed through real-time RT-PCR. (C) Rosette leaf numbers of Ws, *ld-3*, *shb1-D*, and *shb1-D*/*ld-3* under LD and SD. Means plus or minus the standard errors were calculated from at least 15 plants. (D) Expression of *FLC* in Ws, *ld-3*, *shb1-D*, and *shb1-D*/*ld-3* under LD and SD. Expression data are presented as means plus or minus the standard errors from three biological samples and each biological sample was examined in triplicate.

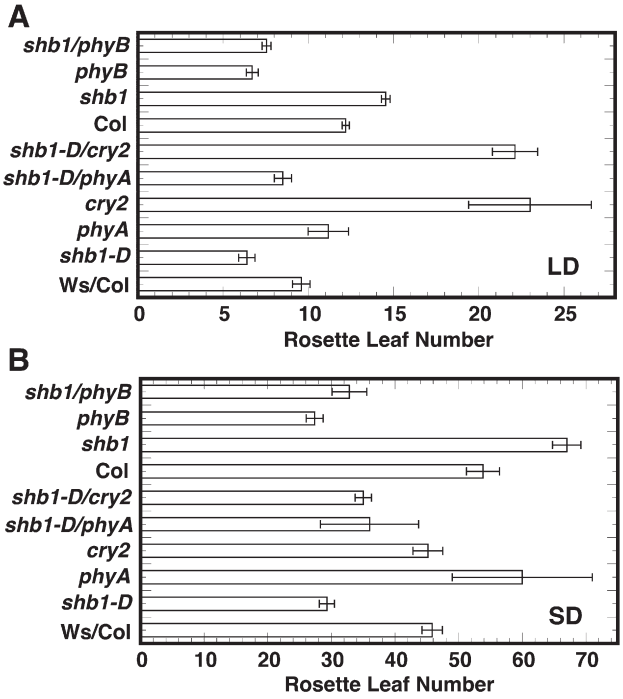

Fig. 7. SHB1 interacts genetically with photoreceptors. (A) Rosette leaf numbers of Ws/Col, *shb1-D* (Ws/Col), *phyA-211* (Ws/Col), *cry2-1* (Ws/Col), *shb1-D*/*phyA-211* (Ws/Col), and *shb1-D*/*cry2-1* (Ws/Col), Col, *shb1*, *phyB-9*, *shb1*/*phyB-9* under LD and SD (B) when bolting 1 cm. Means plus or minus the standard errors were calculated from at least 15 plants.

shb1-D with *phyA-211*, *phyB-9*, and *cry2-1* under long days and short days (Fig. 7). *shb1-D* flowered earlier than did Ws/Col wild type, whereas *phyA-211* flowered slightly later than did Ws/Col wild type under both long days and short days (Fig. 7). The *shb1-D*/*phyA-211* double mutant showed an intermediate flowering phenotype compared to *shb1-D* and *phyA-211* single mutants under both long days and short days (Fig. 7). *cry2-1* flowered much later that did Ws/Col wild type under long days, but flowered normally as wild type under short days (Fig. 7). The *shb1-D*/*cry2-1* double mutant showed a late flowering phenotype like that of *cry2-1* under long days, but an early flowering phenotype like that of *shb1-D* under short days (Fig. 7). Similarly, *shb1*/*cry2-1* showed a *cry2-1*-like flowering phenotype under long days but an *shb1*-like flowering phenotype under short days (Fig. S1).

shb1 flowered notably later than did the Col wild type, whereas *phyB-9* flowered much earlier than did Col under both long days and short days. The *shb1*/*phyB-9* double mutant flowered as early as the *phyB-9* single mutant in both long days and short days (Fig. 7). *shb1-D* flowered early under both long days and short days, whereas *phyB-9* flowered earlier than did *shb1-D* under long days but similarly as did *shb1-D* under short days (Fig. S1). *shb1-D*/*phyB-9* showed a *phyB-9*-like flowering phenotype under long days but an additive flowering phenotype under short days (Fig. S1).

Discussion

SHB1 activates CO and FT to control flowering under long days

Day-length sensing and CO activation occur in the leaves, and subsequently the activated FT protein moves through the pholem to shoot apical meristem where it physically interacts with FD to promote floral initiation (An et al., 2004; Abe et al., 2005; Wigge et al., 2005; Corbesier et al., 2007). In contrast, *FLC* represses flowering when it is expressed either in the phloem or shoot apical meristem (Searle et al., 2006). As the *shb1-D* mutation affects the expression of both *CO* and *FLC*, we have aimed to sort out the contribution of SHB1 to the photoperiodic and autonomous pathways under different day-

length conditions. Our data suggest that the locally expressed SHB1 in leaves may activate the expression of *CO* and subsequent the induction of *FT* expression under long days (Fig. 8). We also found that CO is epistatic to SHB1 under long days, and the activation of *FT* in *shb1-D* requires a functional CO (Figs. 3A, C). In contrast, *shb1-D*/*co-2* double mutant showed a flowering phenotype similar to *shb1-D* under short days, and the activation of *FT* expression in *shb1-D* was only slightly affected by the *co-2* mutation (Figs. 3A, C). As previously reported, CO either binds to *SOC1* promoter to directly regulate the expression of *SOC1* or up-regulates the expression of *FT* and therefore, the expression of *SOC1* (Hepworth et al., 2002; Yoo et al., 2005). The activation of *SOC1* expression in *shb1-D* was partially affected by the *co-2* mutation under long days but was barely affected by the *co-2* mutation under short days (Fig. 3). SHB1 may deploy a different signaling mechanism, such as the one involving LD and FLC, to regulate the expression of *SOC1* under short days.

SHB1 regulates flowering through FLC under short days

Under short days, the activation of *FT* expression by SHB1 may be operated through LD and FLC (Fig. 8). For example, the expression of *FLC* was down-regulated in *shb1-D* and the activation of either *FT* or *SOC1* expression in *shb1-D* was diminished by bringing the level of FLC to that of the wild type under short days (Figs. 4C, D). In contrast, the increase of *FLC* expression to wild type level only partially affected the activation of either *FT* or *SOC1* expression by the *shb1-D* mutation under long days (Fig. 4D). Consistent with previous reports, *flc-3* mutant flowered slightly late under long days, but much later under short days (Fig. 5A; Michaels et al., 2005). The *shb1*/*flc-3* double mutant showed an intermediate flowering phenotype under long days but an *flc-3*-like flowering phenotype under short days (Fig. 5A). Furthermore, the reduced expression of either *FT* or *SOC1* by the *shb1* mutation was partially reverted to a level comparable to that of the Ws wild type under long days, but reverted completely to a level comparable to that of *flc-3* under short days (Figs. 5B, C).

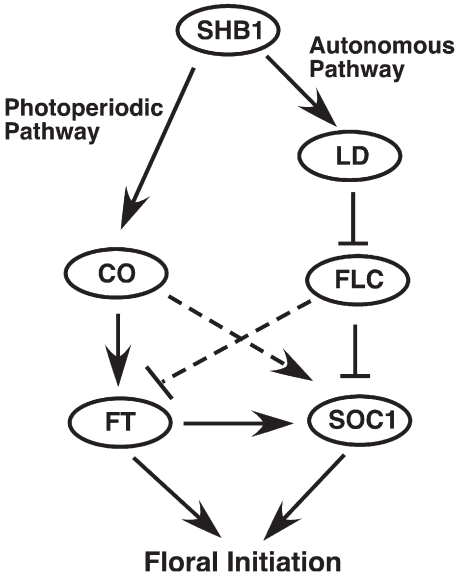

Fig. 8. SHB1 acts in photoperiodic and autonomous flowering as summarized in a simplified model. SHB1 regulates the expression of *CO* and therefore, the expression of *FT* and *SOC1* under long days or inductive photoperiods (left). SHB1 also plays a role in autonomous flowering by activating the expression of *LD* and therefore, the repression of *FLC* expression, allowing the expression of *SOC1* to initiate flowering under short days or non-inductive photoperiods. SHB1 does not directly regulate the expression of either *CO* or *LD*, but may directly regulate the expression of genes upstream of CO and LD. Solid lines indicate the likely paths that SHB1 may act through and the dotted lines indicate the possible routes as suggested by several other studies. Arrows and T-bars represent positive or negative effects, respectively.

SHB1 acts upstream of LD

We have examined the expression of *LD*, *FVE*, *FLD*, *FLK*, *FY*, *FCA* and *FPA* in *shb1* mutants, and only the expression of *LD* was affected by the *shb1* mutations (Fig. 6A). The effect of the *shb1-D* mutation on the expression of *FLC* requires a functional LD under both long days and short days (Fig. 6D). It remains interesting if overexpression of *LD* can mimic the phenotype of the gain-of-function allele *shb1-D* and its effects on *FLC* expression. Interestingly, the expression of *SHB1* overlaps with that of *LD* in shoot apex and floral organs (Fig. 6B, Aukerman et al., 1999). Similarly, *FLC* is also expressed in shoot apical meristem in addition to leaves and root meristem (Sheldon et al., 1999; Noh and Amasino, 2003; Bastow et al., 2004). The overlapping expression pattern of *SHB1* with that of *LD* and *FLC* in the shoot apex may suggest a close functional relation of these three genes. We found that SHB1 is not associated with the promoters of either *CO* or *LD* through ChIP analysis (Fig. S2). We speculate that SHB1 may directly regulate the expression of genes upstream of CO and LD.

Chromatin modifications, including histone acetylation and methylation, play an important role in modulating *FLC* expression (He and Amasino, 2005; Domagalska et al., 2007). The *ld-3* mutation increased the methylation of the *FLC* locus in triMeH3K4 in region IV, corresponding to the 5′ UTR and the first exon (Domagalska et al., 2007). The histone H3 acetylation (H3Ac) was also enhanced in the regions around the translation initiation start, the first exon, and the 5′ region around the first intron of the *FLC* locus in the *ld-3* mutant (Domagalska et al., 2007). Although the chromatin structure of the *FLC* locus in the *shb1* mutants remain to be examined, it is very likely that SHB1 regulate the expression of *LD* and therefore the effects of LD on the chromatin structure of the *FLC* locus.

SHB1 functions downstream of phyB and cry2

The expression patterns of the clock input genes such as *ELF3* and the expression pattern of the central oscillating genes such as *CCA1*, *LHY*, *ELF4*, *TOC1*, and *GIGANTEA* (*GI*) were not altered by the *shb1* mutations compared with wild type (data not shown). *SHB1* transcripts did not show robust oscillation when entrained under 12 h light/12 h dark cycles and SHB1 might not involve in the circadian regulation of the floral initiation (data not shown).

Phytochromes and cryptochromes regulate floral initiation through light quality in a circadian dependent or independent manner. phyB mediates red light repression of flowering under both long days and short days (Mockler et al., 2003; Valverde et al. 2004). phyA promotes flowering possibly through both phyB dependent and independent pathways (Lin, 2000; Mockler et al., 2003; Valverde et al., 2004). In response to extended photoperiods, cry2-deficient *Arabidopsis* plants delay floral initiation by regulating either the abundance of *CO* transcripts or the abundance of CO protein (Guo et al., 1998; Valverde et al., 2004). Our genetic analysis indicate that the effects of the *shb1* mutations on floral initiation require a functional phyB under both long days and short days, and a functional cry2 under long days (Figs. 7, S1). The interaction of SHB1 with phyA appeared more complex.

Accession numbers

Genome Initiative identifiers: *SHB1* (At4G25350), *UBQ10* (At4G05320).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.04.023.

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